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Viability of rat spermatogenic cells *in vitro* is facilitated by their coculture with Sertoli cells in serum-free hormone-supplemented medium

(cell reaggregation/DNA synthesis/spermatogonia/spermatocyte/cell movement)

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ABSTRACT **Spermatogenic cells from 20- to 35-day-old rats were grown *in vitro* in the presence of Sertoli cells maintained in serum-free hormone/growth factor-supplemented medium and alternating high/low concentrations of follicle-stimulating hormone in the medium. In cell reaggregation experiments, spermatogenic cells reassociate with Sertoli cells but not with peritubular cells or cell-free substrate. Autoradiographic experiments using [^{3}H]thymidine as a labeled precursor for DNA synthesis show that spermatogonia and preleptotene spermatocytes, connected by cytoplasmic bridges, have a synchronous S phase. [^{3}H]Thymidine-labeled preleptotene spermatocytes progress until later stages of meiotic prophase. Time-lapse cinematographic studies of Sertoli/spermatogenic cell cocultures show three major movement patterns. While Sertoli cell cytoplasmic processes between adjacent cells display tensional forces, spermatogonia are engaged in oscillatory cell movements different from the nuclear rotation observed in meiotic prophase spermatocytes. Results of this study show that the proliferation of premeiotic cells and the differentiation of meiotic prophase cells do occur *in vitro* in association with Sertoli cells maintained in a medium that allows differentiated cell functions.**

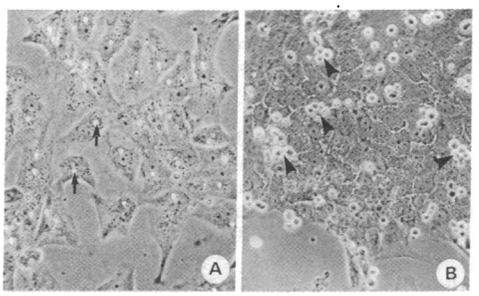

FIG. 1. (*A*) Phase-contrast micrograph of rat Sertoli cells cultured in serum-free hormone-supplemented medium for 15 days. Sertoli cells display cytoplasmic droplets (arrows). Spermatogenic cells present at the time of plating are not present at this time of culture. Tissue culture medium was replaced every day. (×340.) (*B*) Phase-contrast micrograph of spermatogenic cells (arrowheads) obtained from the supernatant of seminiferous epithelial aggregates from a 22-day-old rat. Cells plated for 12 hr reassociate with cultured Sertoli cells as described in *A*. Although a few spermatogenic cells can be seen on the bare substrate, most of them are associated with Sertoli cells. (×240.)

Spermatogenesis *in vitro* has been the subject of numerous investigations (1–6). Although significant progress has been made in the culture of meiotic cells of certain liliaceous plants (7) and spermatogenic cells of *Drosophila* (8) and *Xenopus* (9), attempts to culture dissociated mammalian spermatogenic cells have been relatively unsuccessful. In mammals, the seminiferous epithelium contains spermatogenic cells that maintain spatial and functional relationships with Sertoli cells. Sertoli cell function is regulated by follicle-stimulating hormone (FSH) and androgens (10).

The development of techniques for the isolation and culture of Sertoli cells has facilitated the assessment of several hormone-dependent processes *in vitro* (for review, see ref. 11). Studies in our laboratory have shown that FSH stimulates the synthesis, phosphorylation, and secretion of polypeptides by Sertoli cells (12, 13) when these cells are cultured in serum-free medium supplemented with hormones and growth factors (12). During the course of these studies, it became apparent that clusters of spermatogenic cells remained attached to Sertoli cells despite daily changes of culture medium. This observation provided the basis for the present study.

MATERIALS AND METHODS

Isolation and Coculture of Rat Sertoli/Spermatogenic Cells. Primary cultures were prepared from 20- to 35-day-old rats (Charles River CD) as described (14). Samples of seminiferous epithelial cells were gently suspended in Eagle's minimal essential medium, avoiding disruption of the cytoplasmic bridges connecting spermatogenic cells. Eagle's medium was supplemented with nonessential amino acids (0.1 mM)/4 mM glutamine/1 mM sodium pyruvate/10% fetal bovine serum (Sterile Systems, Logan, UT). Cell samples were plated in 25-cm^2 tissue culture flasks ($\approx 5 \times 10^8$ cells per flask) and on glass coverslips ($\approx 2 \times 10^4$ cells per coverslip). Serum-supplemented medium was changed as soon as cell aggregates attached to the substrate (within 6–12 hr), when it was replaced by serum-free hormone/growth factor-supplemented medium consisting of Eagle's minimal essential medium supplemented as above except for the omission of serum and also with human transferrin (Sigma) at 5 μg/ml, bovine insulin (Sigma) at 5 μg/ml, epidermal growth factor (Collaborative Research) at 3 ng/ml, human growth hormone (AB Kabi, Stockholm, Sweden) at 6.5 international microunits/ml, 10 μM retinol (Sigma), and 0.1 μM testosterone (Calbiochem) (12). After an adaptation period of 24 hr, cells were cultured in serum-free hormone/growth factor-supplemented medium with the above indicated composition except that the concentration of FSH (NIH-oFSH-S14, provided by the Pituitary Hormone Distribution Program of the National Institute of Arthritis, Metabolism, and Digestive Diseases and A. F. Parlow) was increased to 5 μg/ml (high-FSH medium). After 6 hr, the high-FSH medium was removed and replaced with serum-free hormone/growth factor-supplemented medium containing FSH at 0.5 μg/ml (low-FSH medium) in addition to the components already indicated. The low-

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Abbreviation: FSH, follicle-stimulating hormone.

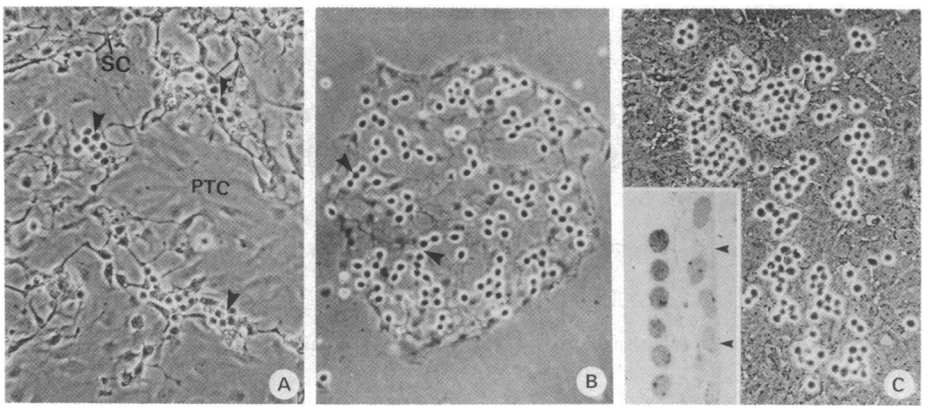

FIG. 2. (*A*) Phase-contrast micrograph showing peritubular cells attached to the substrate (PTC, out of focus). Sertoli and spermatogenic cells were plated on the surface of peritubular cells and allowed to grow for 2 days. The micrograph was obtained after 1 hr of exposure to serum-free hormone-supplemented medium containing FSH at 5 μg/ml. Sertoli cells (SC) display a stellate shape. Spermatogenic cells (arrowheads) are associated with morphologically altered Sertoli cells. Peritubular cells neither adopt a stellate morphology nor show binding sites for spermatogenic cells. (×320.) (*B*) Colony-like formation of stellate Sertoli cells and associated spermatogenic cells arranged in small clumps and in chain-like arrays (arrowheads). Cells were cultured in serum-free hormone-supplemented medium (FSH, 0.5 μg/ml) for 18 hr after plating. (Phase-contrast micrograph; ×320.) (*C*) Coculture as in *B* after culture in serum-free hormone-supplemented medium (high FSH/low FSH) for 8 days. Large spermatogenic cell aggregates, some of them containing >15 cells, can be observed on the surface of cultured Sertoli cells. (×360.) (*Inset*) Section perpendicular to the culture substrate (arrowheads) showing the relationship between columnar-shaped Sertoli cells and a linear array of spermatogenic cells on the surface of Sertoli cells. Cocultured cells were fixed in glutaraldehyde, postfixed in osmium tetroxide, embedded in Maraglas (Polysciences), and stained with toluidine blue. (×420.)

FSH medium was replaced after 18 hr with high-FSH medium. Further medium changes followed this alternating high-FSH/low-FSH, 6-/18-hr, scheduling for up to 2 wk. Serum-free hormone/growth factor-supplemented medium was freshly prepared every 3 days.

Cell Reaggregation Experiments. Spermatogenic cells remaining free in the medium of cultured Sertoli cells were collected within 24–48 hr after plating and used immediately for somatic–spermatogenic cell reaggregation experiments at a concentration of $\approx 1 \times 10^4$/ml. Primary cultures of spermatogenic cell-free Sertoli cells were obtained after 15 to 20 days of culture in serum-free hormone/growth factor-supplemented medium. Depletion of spermatogenic cells from Sertoli cell cultures was accomplished by using a low-FSH medium (0.5 μg/ml) replaced every day. Cultured Sertoli cells have been characterized by several criteria (12–16).

Isolation and Culture of Peritubular Cells. Primary cultures of peritubular cells were prepared as described by Hutson and Stocco (17).

Autoradiographic Analysis of [^{3}H]Thymidine-Labeled Cells. Seminiferous epithelial cell clusters were plated on 18 × 18 mm glass coverslips in 60-mm plastic culture dishes and maintained as described above. Cells were labeled with [^{3}H]thymidine (0.1 μCi/ml; 1 Ci = 37 GBq; specific activity, 60 Ci/mmol; Schwarz/Mann) for 24 hr after 2 to 4 days of plating. Coverslips were fixed after 1, 3, 8, and 24 hr of labeling in methanol/glacial acetic acid (3:1) for 15 min. The radioactively labeled medium was changed to unlabeled medium after 24 hr and additional coverslips were fixed every 24 hr. The experiment was terminated 15 days after plating. Coverslips were mounted cell side upward on microscope slides with Permount (Fisher) and dipped in Kodak NTB-3 Nuclear Track Emulsion. Slides were exposed for 24 hr at 4°C, developed in Kodak D-19 for 30 sec, and fixed in Kodak fixer for 5 min. Slides were stained with 1% aqueous Giemsa for 1 min.

Identification of Spermatogenic Cell Types. Spermatogonial types were identified according to morphological (18) and autoradiographic (19) criteria. Meiotic prophase spermatocytes were identified by their nuclear size and chromosomal patterns (20).

Time-Lapse Cinematography. Movement characteristics of Sertoli and spermatogenic cells were recorded during a culture period of 2 hr to 15 days. Sertoli/spermatogenic cells grown on 25-mm round coverslips were maintained in serum-free hormone/growth factor-supplemented medium following the high-FSH/low-FSH schedule. Studies were carried out with an Opti-Quip 16-mm system (Opti-Quip, Highland Mills, NY) coupled to an inverted phase-contrast microscope (Wild M40A). Cells

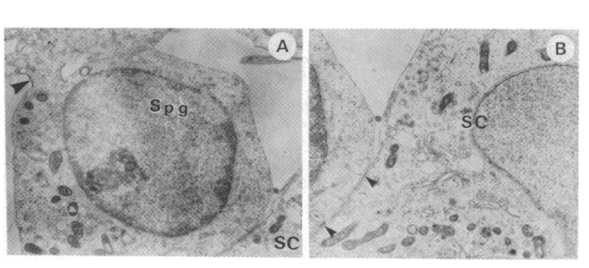

FIG. 3. Cells isolated from a 22-day-old rat were cultured as in Fig. 2*C* for 6 days. (*A*) Electron micrograph showing the attachment of a spermatogonium type A (Spg) to the surface of a subjacent Sertoli cell (SC). The arrowhead indicates an intercellular bridge between two spermatogonia of the same type. (×3,500.) (*B*) Higher magnification (×5,800) of the attachment site (arrowheads) between the Sertoli cell and spermatogonia shown in *A*.

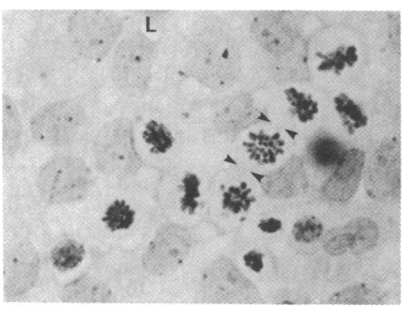

FIG. 4. A Sertoli/spermatogenic cell coculture was maintained in serum-free hormone-supplemented medium (high FSH/low FSH) for 6 days. A group of mitotic cells identified as spermatogonia in a clone-like organization, connected by intercellular bridges (some of them indicated by arrowheads), is seen on the surfaces of cultured Sertoli cells with cytoplasmic lipid droplets (L). (Acetic acid/orcein; ×450.)

were placed in a Dvorak-Stotler (Baltimore Instruments) controlled environment culture chamber. The temperature was maintained at 32°C. Kodak Technical Pan negative film 2415 was exposed with a framing rate of 6 to 120 frames per min during periods of 2 to 7 hr. Film frames excerpted from 16-mm film were printed on photographic paper to quantitate morphological changes.

RESULTS

Spermatogenic Cells Bind to Sertoli Cell Surfaces but Not to Peritubular Cells or Cell-Free Substrate. Experiments were designed to determine whether spermatogenic cells *in vitro* require the presence of Sertoli cells for survival. To test this possibility, spermatogenic cells, identified and quantified in acetic acid/orcein-stained preparations of representative samples, were placed on the surface of Sertoli cells devoid of spermatogenic cells (Fig. 1*A*). After 6–18 hr, most of the spermatogenic cells attached to Sertoli cell surfaces but not to the substrate (Fig. 1*B*). Spermatogenic cell anchorage was not significantly disturbed by several rinses with fresh culture medium.

We then wanted to determine whether spermatogenic cell reassociation with Sertoli cells was cell specific. Primary cultures of peritubular cells were allowed to reach semiconfluent growth (≈5 days after plating). Sertoli and spermatogenic cells together and spermatogenic cells alone were placed on the surface of peritubular cells, allowed to attach, and cultured in serum-free hormone/growth factor-supplemented medium containing FSH at 5 μg/ml. Sertoli cells were identified in the cocultures by their characteristic flat–stellate morphological transition induced by FSH (15) and by the presence of discrete cytoplasmic lipid droplets (Fig. 1*A* and ref. 14). Peritubular cells in culture displayed neither lipid droplets nor morphological changes in response to FSH. While spermatogenic cells remained connected to morphologically altered Sertoli cells (Fig. 2*A*), spermatogenic cells could not be seen associated with the cell-free substrate or with morphologically unchanged peritubular cells.

We then examined spermatogenic cells that had grown on the surface of Sertoli cells for 18 hr (Fig. 2*B*) or 8 days (Fig. 2*C*) after plating. We noticed that, 1 hr after culture in serum-free hormone/growth factor-supplemented medium, chain-like aggregates of spermatogenic cells (3–7 cells per aggregate) maintained their association with stellate-shaped Sertoli cells (Fig. 2*B*). After 8 days, Sertoli cells reached a confluent growth pattern and the relative number (5–16 cells per aggregate) of spermatogenic cells had increased (Fig. 2*C*). When 6-day-old Sertoli/spermatogenic cell cocultures were examined in fixed and stained preparations, we not only confirmed the intercellular links between cells of the same progeny (Fig. 3) but also noted that spermatogonia within a cell aggregate were at the same phase of their mitotic cycle (Fig. 4). This finding suggested that spermatogonia were still capable of synchronous cell division 6 days after plating. If this assumption is correct, pulse-labeling experiments with [^{3}H]thymidine should detect a synchronous S phase in spermatogonia within a single cell aggregate.

DNA Synthesis Is a Synchronous Event Within a Progeny of Cultured Spermatogenic Cells. An autoradiographic approach was used for evaluation of the proliferation and differentiation of rat spermatogenic cells in cocultures. Cells were labeled for 24–48 hr with [^{3}H]thymidine 2–4 days after plating and examined between 1 hr and 11 days after labeling. Fig. 5 illustrates (*i*) the time course of [^{3}H]thymidine-labeled spermatogonia and spermatocytes at leptotene, zygotene, and pachytene remaining attached to Sertoli cells and (*ii*) the percentage of total spermatogenic cells ([^{3}H]thymidine-labeled and unlabeled cells) attached to Sertoli cells during the course of the experiment. The percentage of total spermatogenic cells decreased from 75% at the time of labeling to 38% 11 days later. This finding indicates that, in spite of the improvement of culture conditions, there is still a loss of spermatogenic cells.

After 1 hr of [^{3}H]thymidine labeling, Sertoli cells and spermatogonia displayed silver grains over nuclei (Fig. 6*A*). All nuclei within a spermatogonial cell aggregate showed nearly the

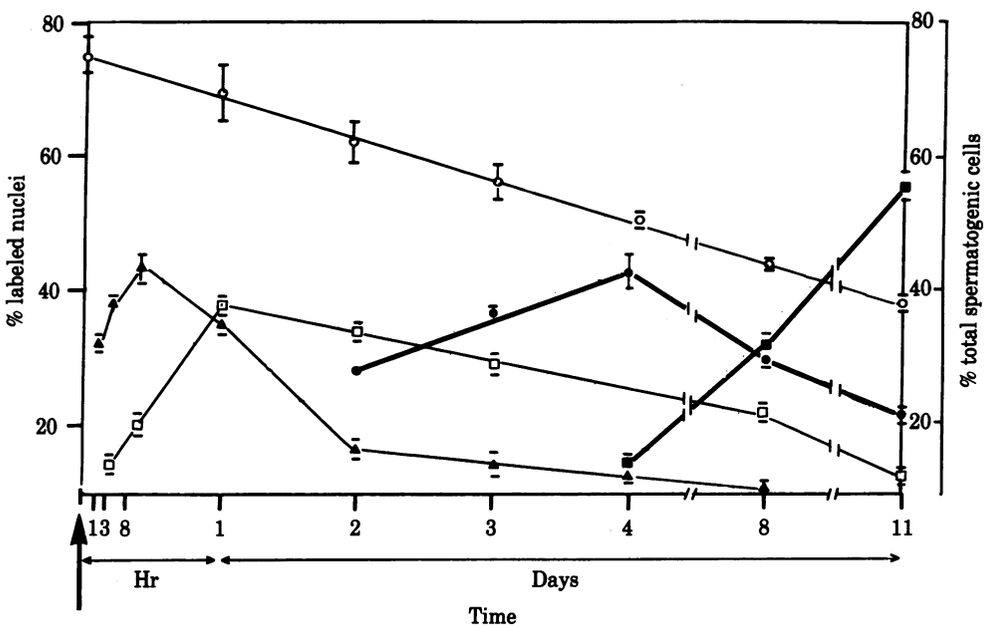

FIG. 5. Time course of [^{3}H]thymidine-labeled spermatogenic cells cultured in the presence of Sertoli cells. Cells were isolated from 20- to 22-day-old rats. "Time zero" (arrow) corresponds to the time when [^{3}H]thymidine (0.1 μCi/ml) was added to the medium (4 days after plating). Percentages of [^{3}H]thymidine-labeled cells (▲, type A and B spermatogonia; □, leptotene; ●, zygotene; ■, pachytene spermatocytes) were recorded at the indicated time intervals. Results represent mean ± SEM of four experiments. ○, Percentage of total spermatogenic cells ([^{3}H]thymidine-labeled and unlabeled) attached to Sertoli cells. Cocultures were maintained in serum-free hormone-supplemented medium following the high-FSH/low-FSH scheduling. Actual rates of cell loss or differentiation are not represented.

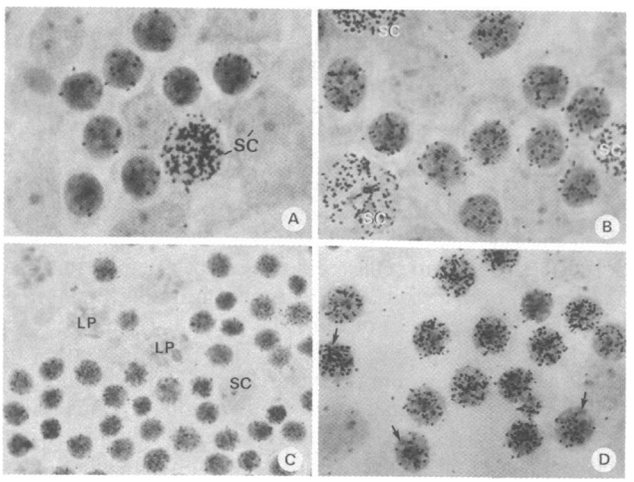

FIG. 6. Autoradiograms of Sertoli/spermatogenic cell cocultures labeled with [^{3}H]thymidine. Cells were isolated from 20- to 22-day-old rats. Cultures were maintained in serum-free hormone-supplemented medium and labeled 4 days after plating. Cells were stained through the photographic emulsion with 1% aqueous Giemsa for 1 min. Nuclear staining was kept to a minimum so that nuclear structures for identification of cell types could be recognized. Therefore, the cytoplasm appears weakly stained or unstained. (*A*) Cells were labeled for 1 hr and fixed in methanol/acetic acid. Silver grains are observed on type B spermatogonia nuclei. Cells are interconnected by cytoplasmic bridges. [^{3}H]thymidine-labeled and unlabeled Sertoli cell nuclei (SC) can be seen in the field. (×600.) (*B*) Cells were labeled for 24 hr and fixed in methanol/acetic acid. Labeled leptotene spermatocytes, identified by the visualization of chromosomal threads, maintain a chain-like arrangement. Silver grains are observed on some SC. (×590.) (*C*) Cells were labeled for 24 hr and fixed 3 days later. Although silver grains are observed on a large number of early pachytene spermatocytes, late pachytene spermatocytes (LP) present in the same field are unlabeled. (×370.) (*D*) Cells were labeled for 24 hr and fixed 11 days later. Arrows indicate the XY chromosomal pair of middle/late pachytene spermatocytes, displaying silver grains over the autosomal bivalents. (×530.)

same number of silver grains, indicating the synchronous nature of S phase. Preleptotene spermatocytes were labeled after a 1-hr [^{3}H]thymidine-labeling period (data not shown), and leptotene spermatocytes were labeled after a 24-hr period (Figs. 5 and 6*B*). Zygotene spermatocytes displayed nuclear silver grains 48 hr after labeling (Fig. 5). Early pachytene spermatocytes showed silver grains 3 to 4 days after labeling, in contrast to the absence of silver grains over late pachytene spermatocyte nuclei present in the same field (Fig. 6*C*). By day 11, a large number of labeled cells had become middle/late pachytene spermatocytes (55%, Fig. 5) displaying the condensed XY bivalent (Fig. 6*D*). Six-day-old Sertoli/spermatogenic cell cocultures established from 35-day-old rats showed an even number of interconnected cells

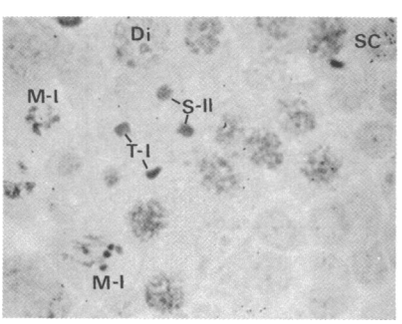

FIG. 7. Transition of metaphase I (M-I) spermatocytes to early telophase I (T-I) and late telophase I (secondary) spermatocytes (S-II) in a 6-day-old Sertoli/spermatogenic cell coculture. Cells were isolated from a 35-day-old rat and labeled with [^{3}H]thymidine for 48 hr 4 days after plating. Di, diplotene spermatocyte. (×430.)

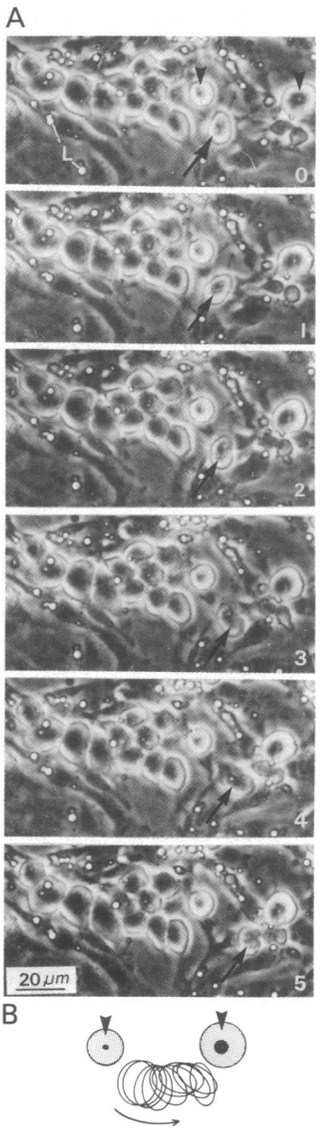

FIG. 8. (*A*) Time-lapse cinematographic sequence showing migration of a spermatogonium (arrow) on the surfaces of Sertoli cells at a speed of 1.5 μm/min, covering a distance of 40 μm. The clear refractile circles are lipids in subjacent Sertoli cells (L). (*B*) Relative position of the migrating cell with respect to two stationary cells (arrowheads). The time in min is given in each print.

in close proximity to metaphase I/anaphase I spermatocytes, suggesting that these cells are secondary spermatocytes and that meiotic division I can be completed *in vitro* (Fig. 7). This finding suggests that cells that had completed S phase before the time of labeling had also completed meiosis I.

Similar [^{3}H]thymidine-labeling experiments were carried out in Sertoli/spermatogenic cell cocultures maintained in serum-supplemented medium. Although spermatogenic cells displayed comparable [^{3}H]thymidine-labeling patterns during the first 4 days, the progression of labeled cells toward more advanced stages of development was impaired by the rapid depletion of spermatogenic cells from the cocultures.

Sertoli Cells, Spermatogonia, and Spermatocytes *in Vitro* Exhibit Distinctive Movement Patterns. We next determined (*i*) the time course of spermatogonia and spermatocyte movement patterns (21, 22) as useful features for identification and characterization of spermatogenic cell types in cocultures and (*ii*) the possible contributions of subjacent Sertoli cells to spermatogenic cell movements. The movement patterns fall into three

major categories. (*i*) Spermatogonia displayed rapid cell oscillating movements of variable velocity (0–0.8 μm/sec) and without coordination within members of the same spermatogonial progeny, (*ii*) meiotic prophase spermatocytes showed clockwise/counterclockwise nuclear rotation. Nuclear rotational movements proceeded along different axes of the nucleus at an average speed of 1.2 μm/sec ($\pm$0.8), and (*iii*) Sertoli cells and peritubular cells showed contractile activity of contacting cytoplasmic processes that did not contribute to or modify the characteristic movement of spermatogenic cells present in the same field.

In some instances, single spermatogonia dissociated from a cell aggregate ceased their oscillations and initiated active ameboid migration on the surface of subjacent Sertoli cells (Fig. 8), later establishing contact but not intercellular bridges with spermatogenic cells in the same field.

DISCUSSION

Aggregates of spermatogenic cells linked by intercellular bridges bind to FSH-responsive Sertoli cells cultured in serum-free hormone/growth factor-supplemented medium. Under these culture conditions, proliferation of spermatogonia and differentiation of meiotic prophase spermatocytes into secondary spermatocytes occur *in vitro*. Cell–cell association between Sertoli cells and spermatogenic cells is a cell-specific phenomenon; neither peritubular cells nor cell-free substrate areas in the presence of Sertoli cell-conditioned serum-free medium could sustain the attachment and long-term continuous survival of spermatogenic cells. This conclusion is also supported by (*i*) the finding in cultures of rat seminiferous tubules that spermatogenic cells do not remain viable on the surfaces of peritubular cells or on bare substrate areas but survive on the surfaces of Sertoli cells (1), (*ii*) the reassociation of specific types of rat spermatogenic cells separated by velocity sedimentation with cultured Sertoli cells but not with dermal fibroblasts (23), (*iii*) the concanavalin A-induced attachment of mouse spermatogenic cells to Sertoli cells *in vitro* (24), (*iv*) the reorganization of dissociated rat spermatogenic cells and testicular somatic cells in rotation cultures into testicular-like tubular structures (25), and (*v*) the FSH-induced modification of rat Sertoli cell surface glycoproteins that may be involved in cell recognition or adhesion (26).

It appears that the combination of an alternating high-FSH/low-FSH scheduling protocol along with other components of the hormone/growth factor-supplemented medium acts synergistically to maintain differentiated functions of Sertoli cells that, in turn, facilitate increased viability of spermatogenic cells and their progression into subsequent stages of development.

Most spermatocytes in a single cell aggregate displayed similar [^{3}H]thymidine-labeling patterns as they progressed into later stages of development. This finding suggests that spermatogonia or spermatocytes associate at the time of plating, remain associated during S phase, and remain connected thereafter as a synchronous differentiating clone. Similar synchronous development of rat (18) and mouse (27, 28) spermatogenic cells has been reported *in vivo*.

Most DNA synthesis *in vivo* occurs in spermatogonia and preleptotene spermatocytes (28). A distinctive but small amount of DNA [0.4% of the genome (29)] is synthesized at zygotene/pachytene in meiotic prophase cells (7, 30) and no DNA synthesis takes place in spermatids (28). It can be argued that *in vitro* uptake of [^{3}H]thymidine into spermatocytes beyond preleptotene may reflect "unscheduled" DNA synthesis in response to cell damage (31, 32) or during *in vitro* incubation (30). Several observations argue against this interpretation. First, when cocultures are labeled with [^{3}H]thymidine for 1 hr, spermatogonia and preleptotene spermatocytes are the only spermatogenic cells displaying nuclear silver grains. Other meiotic prophase spermatocytes present in the cocultures remain unlabeled at this time. Second, the time course of [^{3}H]thymidine labeling of preleptotene spermatocytes shows that the labeled cells advanced into subsequent meiotic prophase stages on schedule. The reduced amount of DNA synthesis that normally occurs at zygotene/pachytene is generally undetected by autoradiography (33). In addition, the possibility of unscheduled DNA synthesis due to cell damage was avoided by labeling cocultures 2 to 4 days after plating. Third, as an indication of persistent cell viability, time-lapse cinematographic analysis of spermatocytes cocultured with Sertoli cells showed that the nuclear rotation observed immediately after plating persisted throughout the course of the experiment.

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